Exhibit 1: Kunkel et al., *Proc. Natl. Acad. Sci. USA* 76(12):6331-6335 (1979)

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Single-strand binding protein enhances fidelity of DNA synthesis *in vitro*

(accuracy/DNA polymerase/DNA binding protein/base selection)

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ABSTRACT **The effect of *Escherichia coli* single-strand binding protein on the accuracy of *in vitro* DNA synthesis has been determined by using two independent methods. By using the synthetic polynucleotide poly[d(A-T)] and measuring dGTP misincorporation or by using ϕX174 DNA and measuring nucleotide substitutions, we found that binding protein increases the fidelity of DNA synthesis by as much as 10-fold. This increase is observed with DNA polymerases of divergent sources and is progressive with increasing concentration of binding protein. The increased accuracy observed with DNA polymerases lacking a 3′ → 5′ exonuclease points to a mechanism other than augmented proofreading. In accord with the properties of single-strand binding proteins, it is suggested that increased fidelity is a result of enhanced base selection by the DNA polymerase, resulting from increased rigidity of the template due to its interaction with binding protein.**

On the basis of spontaneous mutation rates, the accuracy of DNA replication *in vivo* is estimated to be 10^{-7} to 10^{-11} stable misincorporations per base pair (1). This accuracy is several orders of magnitude greater than that measured with purified DNA polymerases *in vitro*, typically 10^{-3} to 10^{-5} (2, 3). The cellular mechanisms used to achieve this enhanced fidelity are unknown, although several mechanisms have been suggested (3, 4). In our continuing effort to understand the cellular mechanisms for the accuracy of DNA replication, we have begun a systematic study of the influence of proteins known to be required for DNA replication on the fidelity of DNA synthesis *in vitro*. Of particular concern was single-strand binding protein (SSB), first purified by Alberts and Frey from T_4-infected *Escherichia coli* (5) and later from uninfected *E. coli* (6). SSB has been shown to be essential for DNA replication (5, 7–9) and to be involved in the processes of recombination and repair (ref. 10; unpublished results). SSB binds cooperatively to single-stranded DNA and destabilizes helical duplexes, causing a lowering of the melting temperature (5). These characteristics have been used to isolate similar DNA binding proteins from several eukaryotic systems (11–15), although their role in DNA metabolism has not been firmly established.

A role for SSB in modulating the accuracy of DNA replication has been suggested by several *in vivo* studies demonstrating that mutations in the gene for binding protein alter the overall mutation frequency of bacteriophage T_4 (16–18). In addition, during the process of copying poly[d(A-T)] with T_4 DNA polymerase, Cillin and Nossal (19) showed a 30–80% reduction in turnover of noncomplementary nucleotides *in vitro* upon the addition of T_4 gene *32* protein (SSB). Also, Liu *et al.* (9) estimated that the error rate of copying ϕX174 DNA by using the T_4 replication complex (seven proteins), which includes SSB, approaches the mutation rate *in vivo*. We therefore desired to assess directly the contribution of SSB to the accuracy of DNA synthesis. We report here our findings that purified *E. coli* SSB increases the accuracy of *in vitro* DNA synthesis by more than an order of magnitude, measured in two independent *in vitro* assay systems. Thus, a protein—other than DNA polymerase and essential to DNA replication—functions in enhancing base selection.

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MATERIALS AND METHODS

Materials. Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals; New England Nuclear was the source of [α-^{32}P]dATP, [α-^{32}P]dTTP, and [^{3}H]dGTP (11,900 cpm/pmol). Poly[d(A-T)], containing only 1 ± 0.5 mol of dGMP for every 2×10^6 mol of dAMP and dTMP, was synthesized as described (20). *E. coli* DNA polymerase (Pol) III was purified by a modification of the procedure of McHenry and Crow (21). Electrophoresis in the presence of sodium dodecyl sulfate confirmed the presence of the α, ϵ, and θ subunits. *E. coli* DNA Pol I (22), avian myeloblastosis virus (AMV) DNA polymerase (23), DNA polymerase-α from acute lymphoblastic leukemia cells (24), and Novikoff hepatoma DNA polymerase-β (25) were purified as described. Calf thymus DNA polymerase-α was a generous gift of Bethesda Research Laboratories (Rockville, MD), and T_4 DNA polymerase was supplied by P. Englund (Johns Hopkins University, Baltimore, MD) and B. M. Alberts (University of California, San Francisco, CA). SSB was purified to >98% homogeneity from *E. coli* strain HMS 83 by the method of Weiner *et al.* (8) or by a simple procedure utilizing blue dextran-sepharose chromatography followed by heating to 100°C (unpublished results). The elimination of any DNA affinity column yields essentially DNA-free preparations of SSB.

Fidelity Assays. Fidelity assays with poly[d(A-T)] as a template were performed as described in the legends to Tables 1–4. In these assays, the error rate is defined as the ratio of incorrect to total correct deoxynucleotides incorporated. The DNA polymerase reactions for copying ϕX174 DNA are as described in the legend to Table 5. A detailed account of the entire methodology for the ϕX174 fidelity assay has been published (26), as was the method used to calculate the error rate from the observed reversion frequency of copied versus uncopied DNA (27).

RESULTS

Fidelity of DNA Pol III. As a first step in assessing the contribution of *E. coli* SSB to fidelity, we measured the accuracy of copying poly[d(A-T)] by purified *E. coli* DNA Pol III. This enzyme was chosen because *E. coli* SSB is required for DNA

Abbreviations: SSB, single-strand binding protein; Pol I and Pol III, DNA polymerases I and III, respectively; AMV, avian myeloblastosis virus.

replication and Pol III has been shown to be the replicative DNA polymerase in *E. coli* (28). Pol III copies poly[d(A-T)] with an error rate of about 1/30,000, using 5 mM Mn^{2+} as a metal activator (Table 1). This accuracy is greater than that reported for Pol III* in copying poly[d(A)]-oligo[$d(T)_{10}$] in Mg^{2+}-activated reactions (30) and less than that reported for Pol III in copying poly[d(A)]-oligo[$d(T)_{12-18}$] with 1 mM Mn^{2+} (31). Synthesis with Mg^{2+} on poly[d(A-T)] was insufficient for quantitation of fidelity. The optimal Mn^{2+} concentration for Pol III (5 mM) was much greater than that reported with other DNA polymerases (20, 23, 29, 32). Moreover, the error rate was not increased at a Mn^{2+} concentration as great as 20 mM (results not given). Omission of Mn^{2+}, dTTP, or, most importantly, poly[d(A-T)] eliminated incorporation of both correct and incorrect nucleotides. Thus, the enzyme preparation was devoid of contaminating DNA, which could act as a template for incorporation of sufficient amounts of dGMP to interfere with any measurements of fidelity. Analysis of the product of the reaction synthesized with DNA polymerases from *E. coli* (Pol I) (20), AMV (23), and human placenta (α and β) (33) indicates that this assay measures internal misincorporation of dGMP present predominately as single-base substitutions.

Due to the high accuracy of Pol III even with Mn^{2+}, any further increase in accuracy would be difficult to quantitate. We therefore defined a set of error-prone conditions for the enzyme by simply increasing the concentration of incorrect nucleotide relative to correct nucleotide substrates in the reaction. As shown in Table 2, the increase in error rate of Pol III is directly proportional to the increase in the concentration of the incorrect nucleotide. These results substantiate previous findings on the effects of biasing substrate pool sizes with Pol I (20, 26), AMV DNA polymerase (23, 29), and T_4 DNA polymerase (19, 32) and are in accord with kinetic models on the fidelity of DNA synthesis (34).

Effect of SSB on Fidelity of Pol III. The results of addition of increasing amounts of SSB to Pol III-catalyzed poly[d(A-T)] assays are shown in Table 3. In experiment 1, equal concentrations of incorrect and correct substrates were used. In experiment 2, the concentration of the incorrect nucleotide was 5-fold greater than that of either correct nucleotide. In both instances, the addition of binding protein in amounts stoichiometric with DNA resulted in a 15–27% increase in the rate of polymerization, whereas greater amounts were inhibitory, as indicated by previous reports (6, 35). Most importantly, SSB increased the accuracy of Pol III-catalyzed DNA synthesis in a concentration-dependent manner. The greatest effect shown here, a 5.8-fold increase in fidelity, was observed at an inhibiting concentration of SSB. This amount of SSB (weight ratio SSB to DNA of 5:1) is slightly less than that required to cover all nucleotides if the template were completely single-stranded. Inhibition of synthesis by greater amounts of SSB prevented quantitation of fidelity. By linear extrapolation of the results obtained with biased pools to the results obtained with equimolar concentrations of incorrect and correct nucleotides, we find that the error rate of Pol III in Mn^{2+} with SSB is less than 1/200,000.

Effect of SSB on Fidelity of Other DNA Polymerases. In order to determine whether the increase in accuracy was limited to a specific reaction of Pol III with *E. coli* SSB, we examined the effect of SSB on the fidelity of DNA synthesis catalyzed by other DNA polymerases (Table 4). The fidelity was increased severalfold with all DNA polymerases studied with either Mg^{2+} or Mn^{2+} as a metal activator. The increase in fidelity observed

Table 1. Fidelity of *E. coli* DNA Pol III in copying poly[d(A-T)]

Condition	Nucleotide incorporated, pmol: Correct	Incorrect	Error rate
I. Complete			
1 mM $MnCl_2$	402	0.001	1/40,200
2 mM $MnCl_2$	624	0.020	1/31,200
5 mM $MnCl_2$	808	0.021	1/38,500
II. Complete			
(5 mM $MnCl_2$)	1785	0.059	1/30,300
− $MnCl_2$	<1.0	<0.001	—
− poly[d(A-T)]	<1.0	0.005	—
− dTTP	2.8	0.008	—

Assays were performed in a 50-μl volume containing 20 mM Tris·HCl (pH 7.5), 5 mM dithiothreitol, 1 μg of poly[d(A-T)], 50 μM each of dATP, [α-^{32}P]dTTP (5–20 dpm/pmol) (1 dpm = 16.7 mBq), and [^{3}H]dGTP (11,900 cpm/pmol), 100 μg of bovine serum albumin per ml, 100 μM ATP, the indicated concentration of $MnCl_2$, and 0.2 (Exp. I) or 0.4 (Exp. II) unit of *E. coli* DNA Pol III. For the experiment in which dTTP was omitted, [α-^{32}P]dATP (5.8 dpm/pmol) was used as the labeled correct nucleotide. Incubation was for either 30 or 60 min at 37°C. Acid-insoluble radioactivity was determined after repeatedly precipitating the polynucleotide product with 1 M hydrochloric acid/0.05 M sodium pyrophosphate and redissolving with 0.2 M NaOH (29). All assays were performed in triplicate and the average incorporation was obtained after subtracting the amount of incorporation in the absence of incubation (typically 15–20 dpm for ^{32}P and 50–100 cpm for ^{3}H).

Table 2. Proportionality of error rate of *E. coli* DNA Pol III to concentration of incorrect nucleotide

dGTP concentration, μM	Nucleotide incorporated, pmol: Correct	Incorrect	Error rate	Relative increase in error rate
50	973	0.029	1/33,600	1.0
100	860	0.055	1/15,600	2.2
175	636	0.065	1/9,780	3.4
250	444	0.068	1/6,530	5.1

Assays were performed as described in the legend to Table 1 with 5 mM $MnCl_2$ and increasing concentrations of [^{3}H]dGTP, as indicated, to a maximum of 250 μM. In each experiment, concentration of dATP and dTTP was 50 μM. All assays were carried out in triplicate. Incorporation in the absence of incubation was determined with each concentration of [^{3}H]dGTP and the values obtained were subtracted. Incorporation of dGTP in the absence of incubation varied from 0.003 to 0.011 pmol at 50–250 μM [^{3}H]dGTP.

Table 3. Effect of *E. coli* SSB on fidelity of *E. coli* DNA Pol III

SSB/DNA*	Nucleotide incorporated, pmol: Correct	Incorrect	Error rate	Relative decrease in error rate
Exp. 1 (50 μM dGTP)				
—	1034	0.032	1/32,000	1.0
0.5:1	1196	0.020	1/59,800	1.9
1.25:1	1314	0.017	1/77,300	2.4
2.5:1	795	0.006	1/132,500	4.1
Exp. 2 (250 μM dGTP)				
—	652	0.086	1/7,580	1.0
0.5:1	750	0.060	1/12,500	1.6
1.25:1	763	0.050	1/15,300	2.0
2.5:1	638	0.028	1/22,800	3.0
5:1	398	0.009	1/44,200	5.8

Assays were performed as described in the legends to Tables 1 and 2 with 5 mM $MnCl_2$ and the indicated amounts of *E. coli* SSB.

* The ratio of SSB to DNA is a weight ratio. Saturation is calculated at 6.6:1, assuming one SSB molecule (74,000-molecular weight tetramer) covers 32 nucleotides (8).

Table 4. Effect of *E. coli* SSB on fidelity of different DNA polymerases

SSB/DNA	Metal activator	Nucleotide incorporated, pmol: Correct	Nucleotide incorporated, pmol: Incorrect	Error rate	Relative decrease in error rate
E. coli DNA Pol I					
—	$MgCl_2$	274	0.011	1/24,900	1.0
0.5:1	$MgCl_2$	527	0.009	1/58,600	2.4
1.25:1	$MgCl_2$	686	0.010	1/68,600	2.8
—	$MnCl_2$	257	0.268	1/959	1.0
1.25:1	$MnCl_2$	410	0.108	1/3,800	4.0
T_4 DNA polymerase					
—	$MgCl_2$	169	0.004	1/42,300	1.0
0.5:1	$MgCl_2$	256	<0.001	<1/256,000	>6.1
1.25:1	$MgCl_2$	334	0.001	1/334,000	7.9
—	$MnCl_2$	195	0.003	1/65,000	1.0
1.25:1	$MnCl_2$	885	0.001	1/885,000	13.6
AMV DNA polymerase					
—	$MgCl_2$	189	0.078	1/2,420	1.0
0.5:1	$MgCl_2$	661	0.075	1/8,810	3.6
1.25:1	$MgCl_2$	626	0.054	1/11,600	4.8
—	$MnCl_2$	751	0.275	1/2,730	1.0
1.25:1	$MnCl_2$	396	0.081	1/4,890	2.0
Novikoff hepatoma DNA polymerase-β					
—	$MgCl_2$	517	0.103	1/5,020	1.0
0.5:1	$MgCl_2$	198	0.014	1/14,100	2.8
1.25:1	$MgCl_2$	47	<0.001	< 1/47,000	>9.4
Calf thymus DNA polymerase-α					
—	$MgCl_2$	198	0.044	1/4,500	1.0
0.5:1	$MgCl_2$	359	0.023	1/15,600	3.5
1.25:1	$MgCl_2$	57	0.001	1/57,000	12.7

Assays were performed in a 50-μl volume containing 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, divalent metal ion activator (2 mM $MgCl_2$ or 1 mM $MnCl_2$), 1 μg of poly[d(A-T)], 50 μM dATP, 50 μM [α-^{32}P]dTTP (5–20 dpm/pmol), 50 μM [^{3}H]dGTP (11,900 cpm/pmol), 0.1–0.5 unit of the indicated DNA polymerase, and *E. coli* SSB as shown. Incubation was at 37°C for 15 min (Pol I) or 60 min (T_4, AMV, α, and β).

with DNA polymerases from AMV, Novikoff hepatoma (β), and calf thymus (α) is not mediated by an increase in excision of incorrectly incorporated nucleotides, because these enzymes lack any proofreading exonucleolytic activity (2, 36, 37). Also, the improvement in accuracy is not correlated with enhanced polymerase activity. Thus, Pol I, T_4 DNA polymerase, and AMV DNA polymerase were stimulated by SSB, whereas DNA polymerase-β was inhibited; yet the accuracy of each of these polymerases was increased. As with Pol III, the increase in accuracy of each enzyme was greatest at the highest concentration of SSB used.

Effect of SSB on Fidelity with a Natural DNA Template. Measurements of fidelity with synthetic homopolymers and heteropolymers are potentially subject to artifacts inherent in the repetitious sequences of these templates. We therefore examined the effect of SSB on the fidelity of DNA polymerases by using the recently developed ϕX174 fidelity assay (26, 27). In this assay, ϕX174 DNA containing an amber mutation in the gene *D/E* overlap is primed with a specific restriction endonuclease fragment and copied by a DNA polymerase *in vitro*. Certain incorrect misincorporations at the amber site will produce reversions to wild type. The DNA is used to infect *E. coli* spheroplasts that are plated on indicator bacteria. The error rate is then determined from the reversion frequency for copied DNA when compared to an uncopied control. Biasing the substrate pool by a relative increase in an incorrect nucleotide—in this case, dATP—provides an error-prone condition that can be used for accurate quantitation of increased accuracy (26). The ϕX174 assay can be used to quantitate error rates for any DNA polymerase capable of utilizing single-stranded DNA as a template. The inability of Pol III to copy long stretches of single-stranded DNA (38, 39) thus precludes measurements of fidelity with this enzyme in the ϕX174 assay.

The error rates of five different DNA polymerases in the presence and absence of *E. coli* SSB when a 5-fold excess of incorrect nucleotide was used are shown in Table 5. With each enzyme, SSB increased the accuracy of DNA synthesis severalfold. Similar results were obtained by using a balanced substrate pool (data not shown). As with poly[d(A-T)], the effect was dependent on the concentration of SSB (with Pol I). It should be noted, however, that the magnitude of the increase in fidelity with SSB did vary considerably, depending on the enzyme and SSB preparations used (data not shown). Because the accuracy of DNA polymerases without 3′ → 5′ exonucleases was increased to an extent similar to that of Pol I, it is likely that the effect of SSB is not mediated by enhanced proofreading.

DISCUSSION

The *in vivo* accuracy of DNA replication of 10^{-7} to 10^{-11} appears to be achieved by a multistep process (3). The free energy of discrimination between incorrect and correct base pairs accounts for an error rate of only 10^{-2}. DNA polymerases enhance base selection to error rates of 10^{-3} to 10^{-5}. All the DNA polymerases used in these studies fall into this range. The experiments described here indicate yet another step in approaching *in vivo* accuracy. SSB enhances the accuracy of *in vitro* DNA synthesis in two independent assay systems by at least 10-fold. Thus, fidelity *in vitro* can approach 10^{-6}. The magnitude of the increase correlates closely with *in vivo* studies (16–18) on the mutator and antimutator effects of mutations

Table 5. Effect of SSB on accuracy of DNA polymerases in copying natural DNA

SSB/DNA	Nucleotides per template	Reversion frequency ($\times 10^{-4}$)	Error rate	Relative decrease in error rate
E. coli DNA Pol I				
—	0	0.287	—	—
—	522	3.12	1/459	1.0
2.5:1	655	1.32	1/1,260	2.7
5:1	684	0.916	1/2,070	4.5
7.5:1	573	0.693	1/3,200	7.0
T_4 DNA polymerase				
—	0	0.787	—	—
—	1154	1.44	1/1,990	1.0
5:1	838	0.841	1/24,100	12.1
AMV DNA polymerase				
—	0	0.416	—	—
—	159	22.2	1/60	1.0
7.5:1	253	1.40	1/1,320	22.0
Novikoff hepatoma DNA polymerase-β				
—	0	0.344	—	—
—	491	1.16	1/1,590	1.0
7.5:1	133	0.640	1/4,390	2.8
Acute lymphocytic leukemia DNA polymerase-α				
—	0	0.476	—	—
—	349	1.42	1/1,380	1.0
5:1	283	0.766	1/4,480	3.2

DNA polymerase reactions were performed in siliconized test tubes in a 50-μl volume containing 20 mM Tris·HCl (pH 8.0), 2 mM dithiothreitol, 1.5 mM $MnCl_2$, 500 μM dATP, 100 μM each of dCTP, dGTP, and [α-^{32}P]dTTP (400–1200 dpm/pmol), 0.2 μg of ϕX174 *am3* viral DNA primed at a 5-to-1 molar ratio with *Hae* III restriction endonuclease fragment Z_5, 0.5–1.0 unit of the indicated DNA polymerase, and *E. coli* SSB. Reactions were incubated at 37°C for 5 min (Pol I and T_4), 60 min (α and β), or 120 min (AMV) and stopped by addition of 1 μl of 100 mM EDTA. Incorporation was determined from duplicate 2-μl aliquots and the values expressed as total nucleotides per template were calculated as an average, assuming all molecules are initiated and copied to the same extent. The remaining reaction mixture was used to transfect *E. coli* spheroplasts, which were then plated as infective centers on permissive and nonpermissive indicator bacteria (26). The reversion frequency of *am3* to wild type was then used to calculate the error rate (27). In an effort to increase the sensitivity of the assay, error-prone conditions were used by biasing the substrate pools to contain a 5-fold excess of one incorrect nucleotide, dATP (26). Whereas the error rates for Pol I and AMV DNA polymerase are in agreement with published results (26, 40), the error rates with α, β, and T_4 polymerases quoted here are initial measurements in Mn^{2+}. The actual error rates in Mg^{2+}-activated reactions for the latter enzymes under optimal conditions are yet to be determined.

in T_4 gene 32 (SSB). The increased accuracy is independent of the use of error-prone conditions, because it is observed with either Mg^{2+} or Mn^{2+} and with biased or balanced nucleotide substrate pools.

At this time, no strong conclusions on the mechanism of enhanced accuracy can be formulated. The severalfold enhancement in fidelity observed with DNA polymerases lacking a proofreading exonuclease indicate that this mechanism cannot be the reason for increased accuracy with these enzymes. However, enhanced proofreading could have a role with enzymes exhibiting a 3′ → 5′ exonuclease. Many mechanisms could explain the increase in fidelity; the simplest of these is enhanced base-selection due to a template/SSB interaction, resulting in increased rigidity of the template. The potential importance of this interaction is emphasized by the observation that a Pol I/Mn^{2+} complex orients the glycosylic bond of the incoming substrate to a conformational angle of 90° (41) — that found in double-helical DNA-β. Such an enzyme-mediated conformational change would position the substrate for correct base-pairing with the template. However, in order to maximize such a conformational enhancement in base selection, the nucleotides in the template should be held rigidly. In this instance, it should be more likely that the incoming nucleotide, if incorrect, will be rejected by steric overlap of the hydrogen bonds between template and substrate (42). Thus, by increasing template rigidity, SSB could amplify base selection during DNA synthesis. Consistent with this hypothesis is the fact that the enhancement in accuracy for a given amount of SSB is not an absolute value but is proportional to the relative accuracy of the DNA polymerase used (Tables 4 and 5). Also, this hypothesis is supported by the 30–80% inhibition of noncomplementary nucleotide turnover observed with T_4 DNA polymerase in the presence of T_4 DNA binding protein (19).

In addition to DNA polymerase and SSB, genetic evidence indicates that other proteins contribute to high fidelity (18). Liu *et al.* demonstrated that ϕX174 DNA could be copied by a seven-protein T_4-replicating complex with an accuracy approaching that achieved during ϕX174 replication in *E. coli* (9). The method of analysis presented here can potentially be extended to determine the contribution to fidelity of any putative replicative protein (43). For example, the true replicative form of Pol III, the holoenzyme (44), contains at least three subunits in addition to those present in Pol III (α, ϵ, and θ), the form of the enzyme used here. Potentially, these additional subunits, in conjunction with binding protein as well as other proteins, may allow *in vitro* DNA synthesis to proceed with the observed *in vivo* accuracy.

A number of exogenous agents have been demonstrated to decrease fidelity of copying synthetic polynucleotide templates (refs. 3 and 45; unpublished results) and, most recently, natural DNA (26). Most of these agents have been designated as mutagens or carcinogens. However, until now no additions to the *in vitro* reaction have been found to increase fidelity. Thus, the large enhancement in accuracy of *in vitro* DNA synthesis with SSB is even more striking, and coupled with *in vivo* data (16–18) it strongly suggests that SSB contributes significantly to the accurate replication of genetic information.

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